ABSTRACT

BIOLOGICAL SCIENCES

TOWNSELL, LESLIE C. B.S. SPELMAN COLLEGE, 2012

EXPLORING THE EFFECT OF SEXUAL RECOMBINATION ON NASCENT MULTICELLULAR ORGANISMS

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Dissertation dated July 2016

The transition to multicellularity is a major step in the evolution of complex life. The first steps in this transition are poorly understood because multicellularity evolved long ago, and transitional forms have been lost to extinction. Previous studies developed a novel microbial model system in which simple multicellularity could be evolved *de novo* (Ratcliff *et al.*, 2012). By evolving our snowflake yeast to undergo sexual reproduction we hypothesized that sex created variation in key multicellular traits, which spurs multicellular adaptation. In our 'snowflake yeast' model system, two traits are of central importance: cluster size, and programmed cell death (apoptosis). Apoptosis previously evolved to regulate cluster size, by acting as break points within clusters, allowing them to modify the size and number of multicellular propagules they produce.

In prior experiments, this only develops after yeast have evolved to form large clusters. Prior experiments in the lab demonstrated that the longer snowflake yeast have been evolving, the greater the fitness benefit provided by sex. Here we examine whether this is due to sex creating greater amounts of diversity in the traits of post-sex offspring in more highly evolved multicellular yeast, allowing post-recombination offspring to 'fine tune' their multicellular traits. By using flow cytometry, we collected data on our multicellular traits. By gathering the biomass mean of the cluster size in each population and staining the cells with propidium iodide to determine the apoptotic tendencies of our cells we were able to compare our outcomes to the pre-sex ancestor, and we determined there was no increase in variation. Although apoptosis did not have an increase in variation due to sex, it created a variation in cluster size; the variation was seen in the population W8. This still supports our hypothesis that sex creates variation in multicellular traits, which allows for rapid adaptation.

Keywords: multicellularity; sexual reproduction; experimental evolution of Saccharomyces *cerevisiae* as a model system; fitness; apoptosis; settling selection

EXPLORING THE EFFECT OF SEXUAL RECOMBINATION ON NASCENT MULTICELLULAR ORGANISMS

A THESIS

SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE

BY

LESLIE CYRENE TOWNSELL

DEPARTMENT OF BIOLOGICAL SCIENCES

ATLANTA, GEORGIA

JULY 2016

ACKNOWLEDGEMENTS

First and foremost I would like to thank God. I would like to thank my mom and dad for always believing in me and encouraging me to advance my education and to always chase my dreams. I would also like to thank them for taking care of me and putting me through school for the past 26 years.

I would like to thank Dr. William Croft Ratcliff for accepting me into his lab and for his guidance in my thesis research project the past two years. I especially would like to thank him for his patience and willingness to go above and beyond in helping me thoroughly understand my project and the background research surrounding it. I would like to thank, Jennifer Pentz, for her assistance in learning the lab protocols and being a sounding board for my experiments. I would also like to thank her for her enthusiasm regarding the advancement my project and my progression in my graduate program. I would like to thank my committee members, Dr. Nathan Bowen and Dr. Zhengxin Wang, for participating in my committee and making sure I am successful in my experiments and on schedule. I would like to thank the entire Ratcliff lab for assisting me in experiments and for keeping me entertained during the long hours spent in the lab.

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PI-Propidium Iodide
YPD- Yeast Peptone Dextrose

CHAPTER ONE

INTRODUCTION

The evolution of multicellularity

The transition from unicellularity to multicellularity is a key step in the evolutionary history of large, complex multicellular organisms (Ratcliff et al., 2012; Michod & Roze, 2001; Grosberg & Strathmann, 2007; Kirk, 2005). Unlike other major transitions, which occurred only once, multicellularity has evolved independently at least 25 times from unicellular ancestors throughout evolutionary history. Multicellularity has originated once in the kingdom Metazoa, but multiple times in the kingdoms Plantae and Fungi (Figure 1) (Grosberg & Strathmann, 2007); multicellularity is also evident in the prokaryotic domain bacteria (Grosberg & Strathmann, 2007; Kirk, 2005). Insight into the transition to multicellularity has proven difficult within the field of evolutionary biology because multicellularity first evolved in eukaryotes around 1.1 billion years ago, and some important transitional steps have been lost along the way (Knoll, 2011). Fortunately, due to work with model systems (both extant simple multicellular organisms like the Volvocine green algae and experimentally evolved multicellular lineages), key insight into the earliest steps in this transition have been uncovered (Ratcliff *et al.*, 2015; Butterfield *et al.*, 2014; Hammerschmidt *et al.*, 2014; Ratcliff *et al.*, 2012; Michod, 2007.)

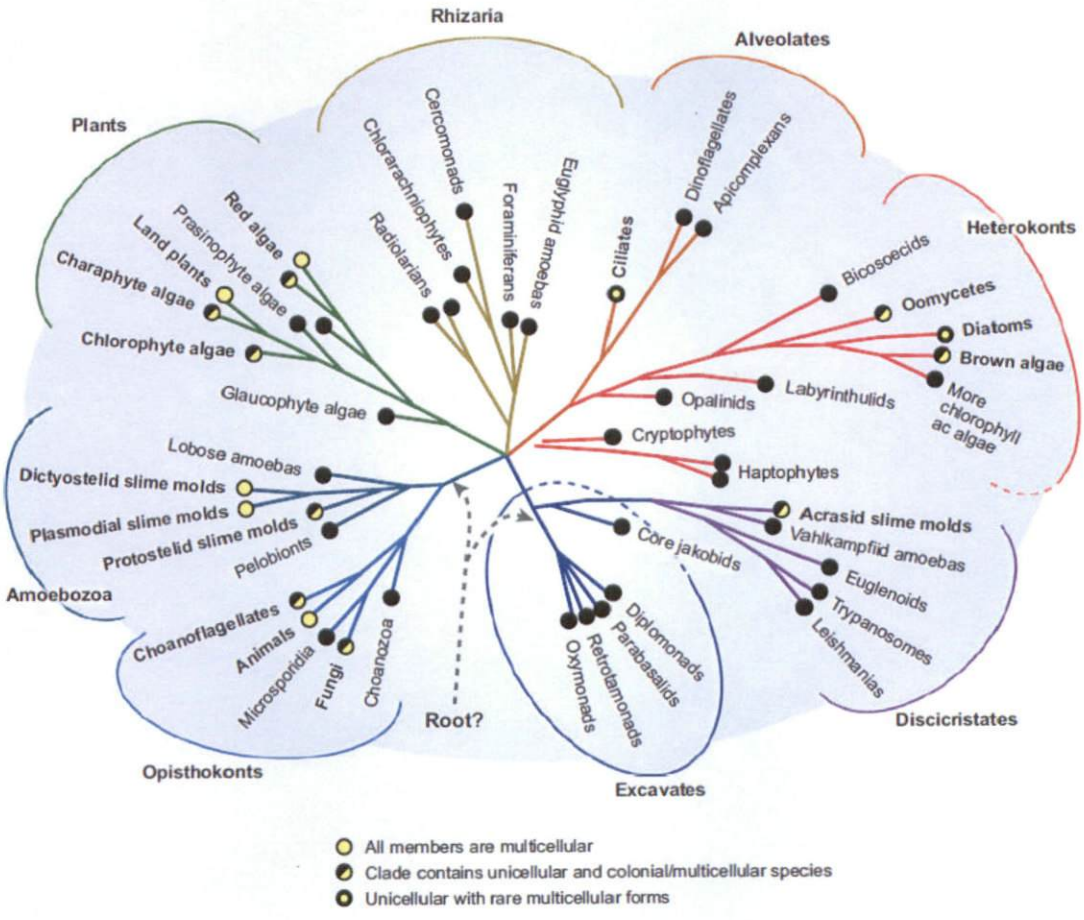


Figure 1. The phylogenetic distribution of multicellularity among eukaryotes. Taxa with yellow circles include only multicellular members, taxa with black and yellow circles include at least some multicellular representatives. (Figure taken from Grosberg & Strathmann 2007).

Multicellular organisms are made up of multiple cells. The first step in the transition to multicellularity was likely the evolution of cellular clusters (Ratcliff *et al.*, 2015; Kirk, 2005; Pfeiffer & Bonhoeffer, 2003). Multicellular clusters can form via two routes: they can grow clonally via incomplete separation after mitotic division, or potentially unrelated cells can form through the aggregation of previously free-living

cells (Ratcliff *et al.*, 2015; Pfeiffer & Bonhoeffer, 2003; Smith & Szathmáry, 1995). The second step in the transition involves a shift in the level of selection-natural selection must begin to act on the traits of whole multicellular clusters (Ratcliff *et al.*, 2015; Pfeiffer & Bonhoeffer, 2003). Third, as a consequence of selection, whole multicellular clusters must respond via multicellular adaptation, or the origination of traits that are adaptive in a multicellular context but not a unicellular one (Ratcliff *et al.*, 2015; Koschwanez *et al.*, 2011; Smith & Szathmáry, 1995). Biological complexity can arise through prolonged multicellular-level adaptation.

There are multiple proposed benefits to becoming multicellular. The first and most frequently invoked potential benefit is that of increased size (Willensdorfer, 2008; Ratcliff *et al.*, 2012, 2013, 2015). Some other benefits of multicellularity include: organism complexity, expanded feeding opportunities, enhanced motility, additional storage reserves (in case of limited nutrients), the creation of internal environments, and the differentiation of cells; the differentiation of cells allows organisms to perform essential functions simultaneously (Dworkin, 1972; Koufopanou & Bell, 1993; Gerhart & Kirschner, 1997; Pfeiffer & Bonhoeffer, 2002; Szathmáry & Wolpert, 2003; Grosberg & Strathmann, 2007; Willsendorfer, 2008; Ratcliff *et al.*, 2012). The advantages of the multicellularity further promoted the transition to multicellularity (Michod & Roze, 2001). The benefits of multicellularity allow for all types of significant advancements, not possible in unicellular organisms (Bonner, 1998). Multicellular organisms exhibit a division of labor, which have allowed them to be successful; tasks such as reproduction and motility can occur simultaneously, unlike in their unicellular ancestor, which had to

choose between the tasks. An interesting example of this cellular division of labor comes from the Volvocine algae. Volvocine algae can partition complementary tasks amongst different cells; they have the capacity to do more work at one time because of the amount of cells that are dedicated to each function, (Michod & Roze, 2001; Kirk, 2005).

The evolution of sexual reproduction

Sex was another major evolutionary transition with profound evolutionary implications (Stearns, 1985). Because sex is constantly occurring, it is notable that the core features: the ability to change from haploid to diploid, the creation of haploid mating partners or gametes from the diploid state, and cell-cell recognition between mating partners or gametes followed by the creation of diploid zygotes, are steadfast, considering how other seemingly important aspects, such as how sex is determined and how sexual reproduction is accomplished is so minor (Heitman *et al.*, 2013; Goodenough & Heitman, 2014). Not all organisms can exist in haploid and diploid phases. *Ulothrix*, green algae, is an example of an organism where mitotic cell divisions and development occur only in the haploid phase; gametes combine to create diploid zygotes, whose meiotic division creates haploid spores. *Fucus*, brown algae, is an example of an organism where mitotic division and development occurs only in the diploid phase; in the haploid phase, single-celled gametes combine to restore the diploid phase (Mable & Otto, 1998). Sexual reproduction exists in all of the major groups of the eukaryotic tree of life (Otto & Leonormand, 2002). It is believed sex appeared one time and has been upheld ever since (Butlin, 2002; Heitman *et al.*, 2013).

Sexual reproduction allows half of each parents genetic material to be transferred to their offspring (Smith, 1978, Otto & Leonormand, 2002). Recombination between each parent's genetic material can result in offspring that are much more variable than either parent's phenotype, as each offspring inherits a unique combination of alleles from the two parents; therefore increasing the fitness of each individual (Rice & Chippindale, 2001). Some models propose that frequent sex and recombination is beneficial (Peck, 1994; Hurst & Peck, 1996). Generally, the benefits of sex accumulate when a portion of the offspring are produced sexually, even a small amount of sex is effective at increasing the rate of recombination of advantageous genes (Green & Noakes, 1995; Hurst & Peck, 1996).

Sex comes at a cost to organisms; despite the cost of sexual reproduction a lot of eukaryotes partake in it (Kleiman & Hadnay, 2015). First, an asexual population has an advantage in fitness over a sexual population where males only add their genetic material for the next generation (Smith, 1971; Levin, 1988; Kleiman & Hadany, 2015). Second, finding partners to reproduce with requires time and energy (Otto & Leonormand, 2002). Lastly, sexual recombination can split current genetic groupings created by natural selection (Barton & Charlesworth, 1998; Otto & Lenormand, 2002; Roze & Michod, 2010; Kleiman & Hadnay, 2015). The cost of sex can increase, linearly, with the amount of offspring produced sexually. Although sex is costly, it is nonetheless an extremely successful evolutionary strategy (Akst, 2010). Similar to *S. cerevisiae*, the rotifer, *Brachionus calyciflorus*, can reproduce both sexually and asexually. Becks and Agrawal, (2010) tracked the rotifer, over the course of 100 generations to test the theory that sex

can evolve, they concluded that the high rate of sex is valuable in heterogeneous environments, therefore negating the high cost (Smith, 1978; Becks & Agrawal, 2010).

The yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*), can reproduce through either budding clonal offspring (mitotic division), or through meiotic sex (Heitman *et al.*, 2013). The method in which yeast determine whether to reproduce asexually or sexually is dependant on the availability of carbon and nitrogen (Smutzer, 2001). Mitotic division, or selfing, in S. *cerevisiae* is a form of sexual reproduction, in which a mother cell can switch mating type and mate with a daughter cell to homozygose the entire genome (Figure 2) (Heitman *et al.*, 2013). More specifically, S. *cerevisiae* cells can either be haploid or diploid, but they prefer to be diploid; both haploid and diploid cells reproduce by mitosis with daughter cells budding off of mother cells (Mable & Otto, 1998). When yeast are haploid they possess one of two mating types: 'a', or 'α'; these can fuse to form a diploid zygote. In the diploid phase, during the sexual life cycle, meiosis takes place and the haploid offspring are produced. The offspring can then reproduce either asexually through mitosis or sexually by repeating the sexual life cycle (Heitman *et al.*, 2013).

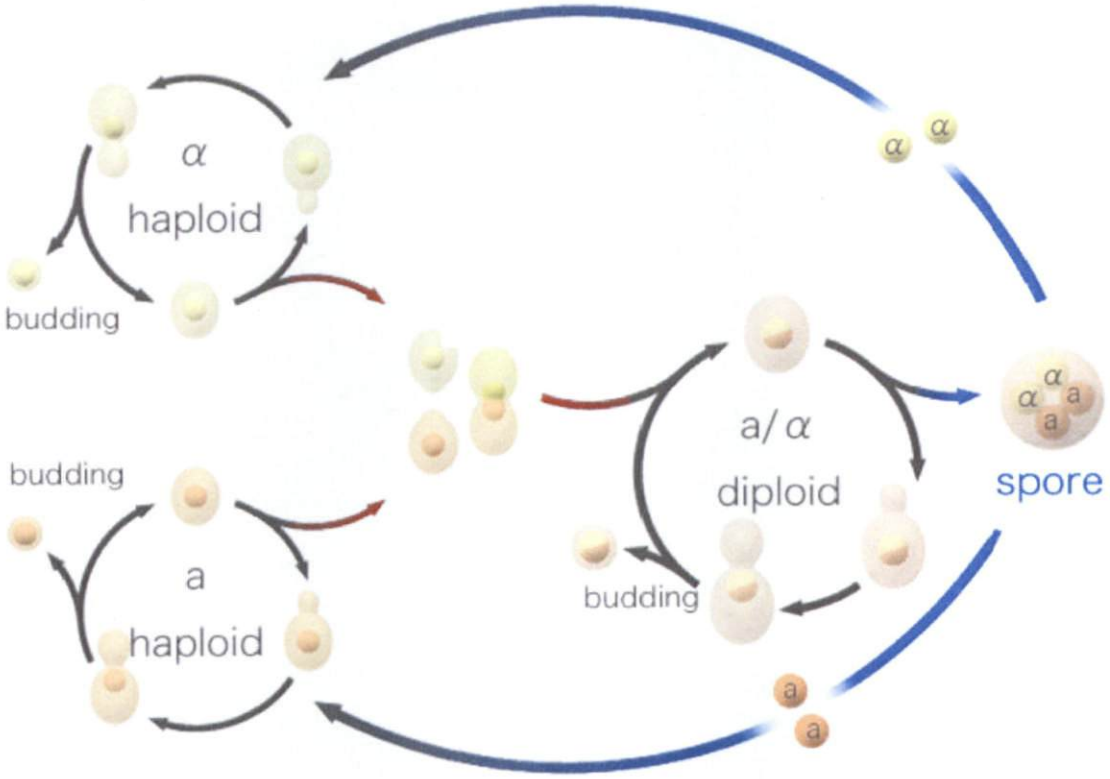


Figure 2. Sexual reproduction in S. *cerevisiae.* Yeast cells can be either haploid or diploid, though they prefer to be diploid. Both haploid and diploid cells reproduce by mitosis with daughter cells budding off of mother cells. When yeast are haploid they can either be 'a' haploid or 'α' haploid. When an 'a' cell is in the presences of a 'α' cell they can mate to become diploid (Figure taken from Asher, 2014).

PURPOSE OF STUDY

The significance of this project is to observe the role of sex in the transition to multicellularity. This study will investigate two specific multicellular traits, cluster size and apoptosis, observed in our evolved snowflake yeast. Using *S. cerevisiae* as the model system we want to determine if sex can create more variation in cluster size and the frequency of apoptosis seen within each cluster. From our findings we want to determine which cluster has the optimal combination of traits in order to increase its fitness level. The goal of this study is to close the gap in our knowledge of the evolution of sex in multicellularity by rediscovering the lost steps in the transition. The results of this study will bring researchers one step closer to understanding how sex plays a role in the initial steps in an organism transitioning from unicellularity to multicellularity. It will help researchers determine if sex promotes the transition to multicellularity or constrains it.

CHAPTER TWO

LITERATURE REVIEW

Background

Experimental evolution of *Saccharomyces cerevisiae* as a model system

S. cerevisiae, or yeast, is a popular model system for eukaryotic biology because it is cheap and can be easily grown and manipulated genetically (Botstein *et al.*, 1997; Smutzer, 2001). Yeast are simple, free-living, unicellular eukaryotes or fungi. Yeast have a chromosomal structure of DNA comparable to the DNA in higher eukaryotes, like mammals; biologists use these simpler systems because of the similarities they share such as membrane-bound organelles and cytoskeletal organization (Botstein, 1991; Smutzer, 2001).

Snowflake yeast as a model system for evolving multicellularity

Ratcliff *et al.*, (2012) used experimentally evolved *S. cerevisiae* to observe the first steps of the transition into multicellularity (Ratcliff *et al.*, 2012; Koschwanez *et al.*, 2011; Boraas *et al.*, 1998). To select for simple multicellularity in, *S. cerevisiae,* Ratcliff *et al.*, 2012 used fast sedimentation through liquid media as a way to select for clusters of cells, as cluster of cells settle faster than single cells. This resulted in the evolution of

clonal clusters termed 'snowflake yeast' (Figure 3). Once snowflake yeast evolved, it was determined that whole clusters either succeed, and get to the bottom of the tube in time, or fail to do so and perish. To see if this results in a shift in the level of selection, from among single cells to among clusters, a divergent selection experiment was performed, selecting for either fast or slow settling from a common ancestor (Ratcliff *et al.*, 2012). Snowflake yeast responded by either evolving a large or small cluster size, indicating that selection is acting at the cluster-level. Because snowflake yeast cells grow quickly, and stay attached to each other poses a problem, the larger the cluster size the slower it grows, which is costly. Asymmetric division allows clusters to produce fast-growing offspring that circumvent this constraint (Ratcliff *et al.*, 2012; Rodriguez-Menocal & D'Urso, 2004). To control the large snowflake yeast cluster size and quick growth rate, elevated apoptosis evolved to mitigate the trade-off between cluster size and growth rate.

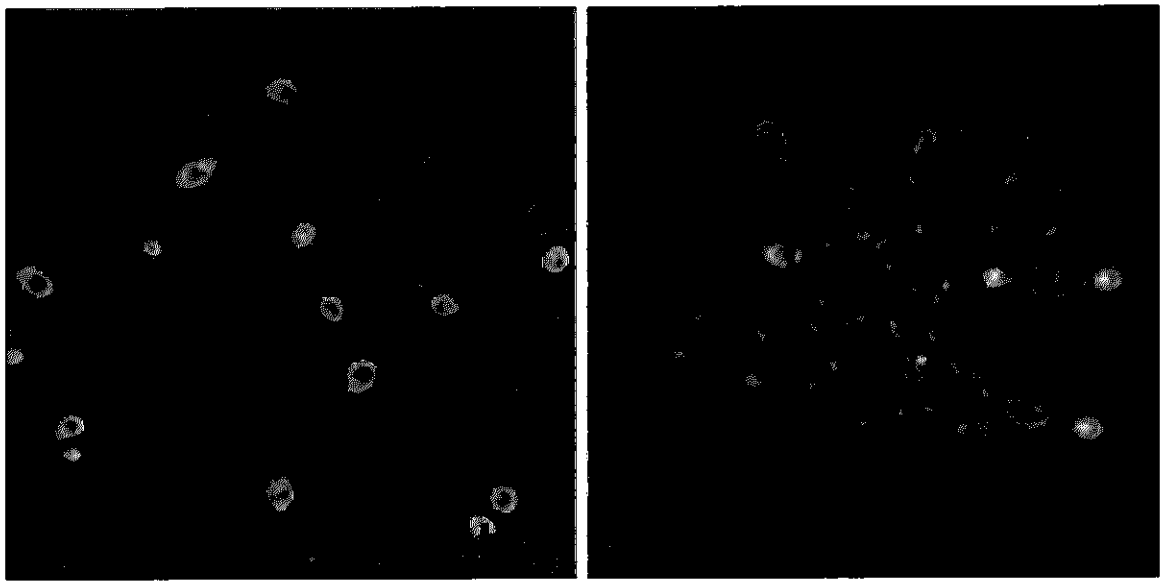


Figure 3. The evolution of the multicellular "snowflake" phenotype. A) Unicellularity is exhibited by the ancestor and B) multicellularity is evident in the cluster formation of individual cells (figure taken from Ratcliff *et al.*, 2012).

Apoptosis

Apoptosis is a controlled, energy-dependent form of cell death important for metazoan development; it plays an important role in protecting organisms, by removing infected cells or cells that have undergone irreparable DNA damage (Concannon *et al.*, 2003; Madeo *et al.*, 2004; Rodriguez-Menocal & D'Urso, 2004). S. *cerevisiae* is a single celled organism with a cellular suicide program (Madeo *et al.*, 2004); apoptosis is commonly seen in single celled organisms, including S. *cerevisiae* (Madeo *et al.*, 1999; Rodriguez-Menocal, 2004; Carmona-Gutierrez *et al.*, 2010; Ratcliff *et al.*, 2012). For snowflake yeast, apoptosis is used as a method of reproduction; apoptotic cells are a break point within clusters, allowing clusters to produce multicellular propagules;

apoptosis can be detrimental to the cell that is performing the act of cellular suicide, but beneficial for the whole cluster.

Through the course of the experiment the rate of apoptosis increased; this is a cluster-level adaptation, benefitting the cluster as a whole. Apoptosis is a type of division of labor, allowing large cluster-forming genotypes of snowflake yeast to make proportionally smaller, faster-growing propagules. (Ratcliff *et al.,* 2012). It plays an important role for the evolution of snowflake yeast, it allows the cluster size to be regulated, and ensures nutrients are able to reach all cells. In snowflake yeast, apoptosis causes the large parent cluster to break a part into smaller daughter clusters by way of cellular separation; this is caused by the separation of apoptotic cells from live cells. Apoptosis is an important adaptation in the evolution of multicellularity, similar to the evolution of sexual reproduction (Ratcliff *et al.*, 2012).

Preliminary Data

To understand the role that sex may play during the initial transition to multicellularity, isolates from each week of the evolution experiment performed by Ratcliff *et al.* (2012) were put through one round of selfing sex to generate diverse post-sex population. Briefly, single strain isolates were put into sporulation media to allow the yeast to undergo meiotic division, generating haploid progeny within a spore. The spore coat was then enzymatically digested, releasing individual haploid progeny, which was then plated onto yeast peptone dextrose (YPD; per liter 20 g dextrose, 20 g peptone, 10 g yeast extract, 15 g agar) at a high dilution so they were separated from each other

allowing them to autodiplodize. Multiple colonies were scraped and put into a tube of liquid YPD creating a diverse selfed population. As a control, this was also done in unicellular populations evolved for the same duration of time with the absence of selection for settling (100 μL of culture was propagated daily to 10 mL of liquid YPD). Pre-sex and post-sex populations were competed against a common competitor to determine relative fitness.

It was determined sex only increases the fitness after the transition to multicellularity. The benefit of sex is dependent on the number of days of evolution for the post-sex population. There is no fitness benefit of sex for our unicellular yeast, which evolved as a unicellular yeast population for the same amount of time (60 days), sex actually creates a small cost for the unicellular ancestor (Figure 4). From this experiment we set out to measure if sex is beneficial to multicellular snowflake yeast and not the unicellular yeast and if that benefit increases the longer yeast have been evolving as multicellular clusters. Fitness is the ability of organisms, populations, or species to survive and reproduce in their current environment; reproduction causes the movement of genetic material from parent to offspring, allowing for the inheritance of successful traits to survive (Orr, 2009). Fitness can also be measured quantitatively; for our experiments fitness was measured as the proportional change in a clone, or allele's, frequency in the population across one generation. All fitness benefits from sex depend on its ability to create diversity in offspring: we thus expect that as snowflake yeast evolve for longer periods of time and gain more multicellular traits, that sex unleashes more variation in

multicellular traits. And, consistent with Fisher's Fundamental Theorem, that the rate of adaptation in these post-sex populations will be proportional to the amount of standing additive genetic variation.

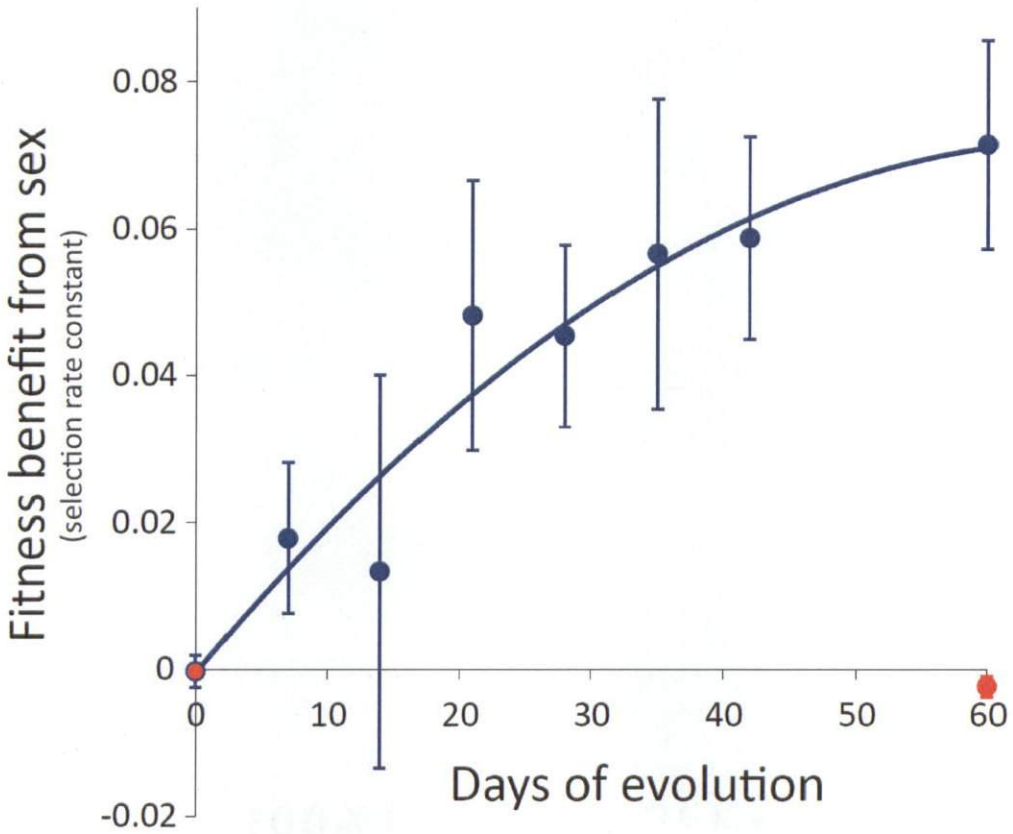


Figure 4. The red dot indicates the unicellular ancestor. The graph shows: the fitness benefit of sex (fitness of the post-sex population-(minus) the pre-sex population, which shows the fitness benefit gained from sex) on the Y-axis and the days of evolution on the X-axis. At zero we have our unicellular ancestor in red and the multicellular ancestor in blue. Above zero shows a benefit of sex, below zero shows a cost of sex. After 60 days sex is costly to the unicellular ancestor causing it to land below zero and sex is beneficial to the multicellular ancestor causing it to land above zero.

Hypothesis

Sex creates variation in multicellular traits, which allows for rapid adaptation. The longer they have been evolving as multicellular organisms, the more variation is unleashed by sex.

Specific Aims

Specific Aim One: Determine if sex creates more variation in multicellular traits in more-evolved snowflake yeast.

Specific Aim Two: Determine which isogenic culture has the optimal level of combined fitness traits, by measuring the size and apoptosis.

Experimental Design

Isolating Single Strains

The post-sex population was diluted using a 1:10,000 dilution to collect individual colonies on petri plates. From these plates, 96 isogenic cultures were chosen at random to show the variation in each of the weeks of evolution. These 96 strains were used as freezer stocks and stored in a -80° freezer.

Growing Single Strains

The 96 strains from each of the 7 weeks of evolution (C1W1S-C1W6S and C1W8S), were plated on YPD agar petri plates and placed in an incubator to grow for two days.

The YPD agar plates were made using1000 mL Di H20, 20 g glucose, 20 g meat peptone, 10 g yeast extract, 15 g agar. After two days of growth, the plates were removed and the cells grown from the 96 isogenic cultures were placed in separate tubes of liquid YPD media. The YPD liquid media was made the same as the agar plates without the inclusion of the 15 g of agar. The tubes were placed in a shaker and the cells grew for 24 hours. After 24 hours the tubes were removed from the shaker and 200µl of liquid media from the old tubes were transferred to fresh media. The new media was placed back in the shaker to grow for an additional 24 hours. After the second round of growth in the shaker, the colonies were then removed and further diluted using a 1:10 dilution.

Flow Cytometry

Flow cytometry was be used to measure two specific traits that may affect cluster level fitness, percent apoptosis and cluster size, in order to look at variation within the post-sex population. A Cube 8 Robby flow cytometer by Partec was used to collect the data. To collect the data ready to use Calibration Beads distributed by Partec were used to align the regions previously set on the machine. Calibration beads were replaced with water and ran through the machine in order to clean it. A script written in MATLAB, which allows for the automatic gating of cells and a more concise fraction of clusters that contain detectable levels of dead cells, was used to capture the specific data. In order to determine the percent dead cells, the samples were stained with Propidium Iodide (PI). The program was run allowing the machine to take up 15µl of the sample. The flow cytometer was cleaned after each sample using water.

Parent Strains

The pre-sex ancestor cells of each population were grown up from freezer samples and underwent the same protocol as the isogenic cultures. This was done in order to compare the parent to the entire population and determine the variation in each population.

Data Analysis

The FCS express program and MATLAB script were used to export and analyze data to determine cluster size and the percent of dead cells in each sample.

Cluster Size

A program was used to export data from the flow cytometer runs into comma separated value (.csv) files. The data was analyzed in a MATLAB script. This script allowed for the collection of the average FSC value of each strain. The collected data was copied into an excel document and used to generate a graph.

Proportion of Clusters Containing Dead Cells

The script allowed us automatically gate our populations into those clusters that contain dead cells (detectable PI signal) vs. those that do not. This allowed us to rapidly screen each recombinant genotype for apoptotic activity. This data was be copied into excel. To determine the percent of dead cells in each sample, the equation: (PI)/(PI+Non PI), was be used to generate a graph.

Anticipated Data and Interpretation

Specific Aim One

In order to ascertain if sex creates more variation in previously evolved snowflake yeast, seven parental genotypes, measured between 7-60 days of evolution, were evolved with sex. We examined the variation in two key multicellular traits: cluster size and apoptosis, in 96 post-sex isolates for each parental genotype. We hypothesized that post-sex offspring from more highly evolved snowflake yeast will be more variable in these key traits.

Specific Aim Two

In order to determine how selection acts on variation created by sex, we performed a selective screen on our populations of meiotic segregates. We competed all 96 post-sex isolates from each parental strain against each other (one competition per parental genotype), and examined which combinations of size and apoptosis are favored by selection. If our hypothesis from specific aim one is supported, and sex increased variation in multicellular traits, then this aim should allow us to determine if selection is acting on this variation. Specifically, we will determine if the multicellular traits (cluster size, apoptosis) change more with selection for highly evolved snowflake yeast parents, suggesting that selection is capitalizing on the variation they create. Thus, the purpose of this aim was to bridge the link between the creation of variation in multicellular trait-space, and the evolutionary consequences of this variation.

Potential Problems and Alternative Strategies

Specific Aim One

In determining if sex can create more variation some of the following potential problems could arose. Because of our large sample size, all the flow cytometer runs did not take place on the same day; this could caused a variation in the way the flow cytometer analyzed our samples from day to day. In order to solve this problem, ten random samples from each population were run together during the same flow cycle, ensuring that the seven populations have samples run under the same conditions.

Specific Aim Two

In determining if selection is acting on variation created in key multicellular traits, size and apoptosis, some of the following problems could arose; five days of selection was not a long enough experimental period causing no variation in traits, in order to solve this, the experiment could be extended to ten days of selection. Another possible problem is new combinations or traits are created, and we only want to select on the current diversity. In order to solve this problem the experiment could be shortened, therefore, decreasing the amount of selection seen.

CHAPTER THREE

MATERIALS AND METHODS

Specific aim one

Liquid media was prepared by using 1000mL DI water, 20 g dextrose, 20g meat peptone, and 10g yeast extract supplied by VWR. Agar media was prepared by using 1000mL DI water, 20g dextrose, 20g meat peptone, 10g yeast extract, and 15g/L agar. Each chemical was weighed in weigh boats using a scale. This media prepared multiple times and was used throughout the course of the experiment.

C1W1S, C1W2S, C1W3S, C1W4S, C1W5S, C1W6S and C1W8S are all single strain isolates. Previously, each isolate was selfed or put through sex. Spores were generated within individual cells and cells were enzymatically digested, freeing individual spores (Figure 5). Isolates from every week of the Ratcliff *et al.*, 2012 evolution experiment were put in sporulation media, which generated tetrad cells. The spore coat was enzymatically digested, freeing the haploid spores. Single spores were germinated on petri plates where they were separated from each other. The cells were plated at 1:500 dilution. The agar plate was placed in a static incubator for 48 hours. After 48 hours multiple colonies were scraped and put into a tube of liquid YPD creating a diverse selfed population or post-sex population. To determine relative fitness, pre-sex

and post-sex populations were competed against a common competitor. Five replicate populations of each pre-sex and post-sex were used.

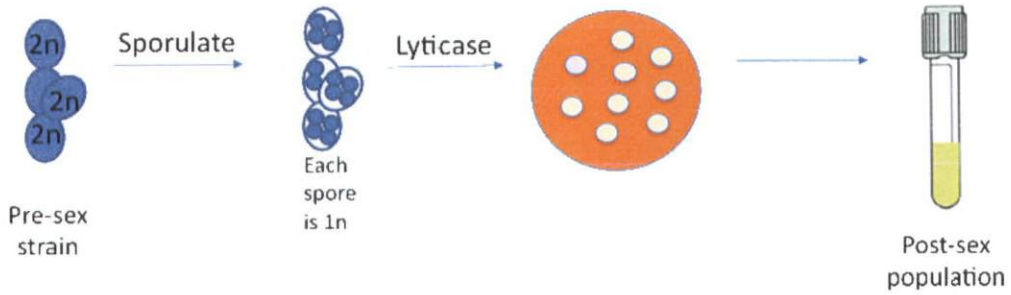


Figure 5. An illustrated depiction of the Ratcliff *et al.*, 2012 experiment. The experiment from Ratcliff *et al.*, 2012 created the selfed yeast populations. This data is essential to isolating and growing isogenic cultures and analyzing post sex population data.

Isolating single strains

Single strain post-sex isolates from the seven populations (C1W1S, C1W2S, C1W3S, C1W4S, C1W5S, C1W6S, and C1W8S) were diluted in a 1:10,000 dilution and streaked on agar plates using glass beads in order to collect individual colonies (Figure 6). The agar plates were placed in a static incubator at 30° C for 48 hours. After 48 hours the plates were removed from the incubator and from these plates, 96 isogenic cultures were chosen at random, by scraping each individual colony into 10mL of fresh YPD media, to show the variation in each of the weeks of evolution. Cryogenic archives of these 96 strains were made and stored in a -80° freezer.

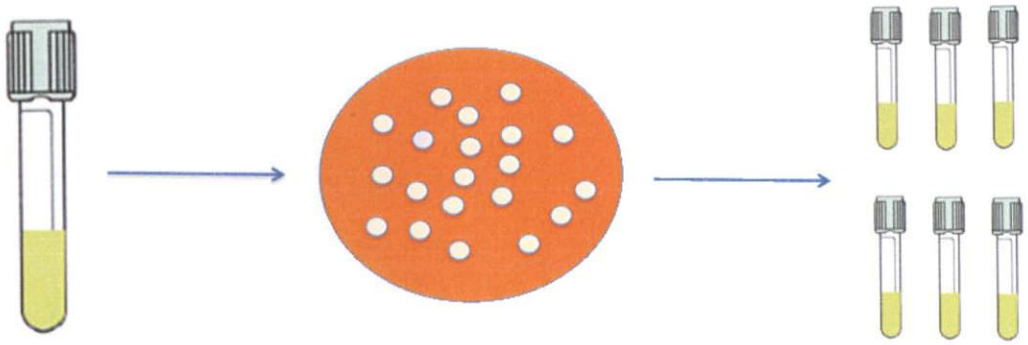


Figure 6. An illustrated depiction of isolating single strain isogeneic cultures. 96 separate post-sex strains per time point were isolated by streaking the populations on petri plates and diluted (1:10,000).

Creating freezer stocks

Freezer stocks were created by putting the 96 isogenic cultures into liquid YPD media. The cultures grew for 24 hours in a shaking incubator. The samples were then transferred to new tubes by vortexing the sample, flaming the top of the tube then removing 200uL of the old YPD media and sample, and placing it in the fresh YPD media. 700uL of the sample was then placed in into a 2mL sterile freezer tube where 300uL of 70% glycerol was also added. These tubes were labeled and stored in freezer boxes in a -80° freezer.

Growing single strains

The 96 strains from each of the seven weeks of evolution, and the pre-sex ancestor cell isolates were plated on YPD agar petri plates and placed in an incubator to grow for two days. After two days of growth, the plates were removed and the cells grown from the 96 isogenic cultures and pre-sex isolates were placed in separate tubes of

liquid YPD media. The tubes were placed in a shaking incubator at 30°C and the cells grew for 24 hours. After 24 hours the tubes were removed from the shaking incubator and 200µl of liquid media from the old tubes was transferred to fresh media. The new media was placed back in the shaking incubator to grow for an additional 24 hours. After the second round of growth in the shaking incubator, the colonies were removed and further diluted using a 1:10 dilution (200uL sample and 1800uL DI water.) The pre-sex ancestor cells of the seven populations were used in order to compare the parent to the entire population and determine the variation in each population.

Flow cytometry to measure cluster size and apoptosis

Flow cytometry was used to measure two specific traits that may affect cluster level fitness, percent apoptosis and cluster size, in order to look at variation within the post-sex population. The flow cytometer has a blue-green light, which excites cells and allows them to re-emit light of varying wavelengths; Snowflake yeast cells are full of protein which auto fluoresce and re-emit light from other spectra. The flow cytometer records the amount of voltage produced from the photo detector. The cluster size is measured based on the amount of autofluorescence the cells produce. The autofluorescence is the brightness of the event; the brightness of the event determines how cells auto fluoresce. The brighter the event, the larger the cluster is.

A Cube 8 Robby flow cytometer by Partec was used to collect the data. To collect the data ready to use Calibration Beads distributed by Partec were used to align the regions previously set on the machine. Calibration beads were replaced with water

and run through the machine in order to clean it. A previously written program, which detects cluster size and the percent dead cells, was used to capture the specified data. In order to determine the percent dead cells, the samples were stained with Propidium Iodide (PI) a red fluorescent stain used to detect dead cells. The program was run allowing the machine to take up 15μl of the sample. Between each sample, water was run to clean the machine.

Specific aim two

Settling selection

The post-sex strains of C1W1S, C1W2S, C1W3S, C1W4S, C1W5S, C1W6S, and C1W8S were removed from the freezer and defrosted. After the post-sex strains defrosted, a micropipettor with wide boar tips was used to pipette 100uL of each population into separate test tubes containing 10mL YPD and 10uL amp. Tubes were placed in the shaker and grown over a period of 24 hours. After 24 hours, the tubes were removed from the shaker, and eight replicate populations of each strain were made. To make the eight replicate populations, 100uL of the population was placed into 10mL of fresh YPD media this was repeated until eight replicate strains were made for each population. The tubes were placed back in the shaker and grown for another 24 hours. 1.5mL of each tube was placed in a 2mL microcentrifuge tube. After all the samples had been transferred into 2mL microcentrifuge tubes, four tubes were vortexed, then spun down in the centrifuge on 100g for 10 seconds, the top 1400uL was thrown out and the

bottom 100uL was placed in 10mL of fresh YPD media. Four tubes were settled at a time until all samples were completed. The new tubes of YPD media were placed in a shaker and grown for 24 hours. The first set of samples (t0) were placed in the fridge and saved until later. The settling was repeated everyday for a total of five days starting with the transfer of 1.5mL of all the samples with the exception of placing the tubes in the fridge to save. On the fifth day of settling the tubes from the fridge were removed and placed into 10mL of fresh YPD media, all the tubes were placed in the shaker and grew for 24 hours. On the last day a 1:10 dilution of the samples were placed in DI water, 20uL of PI was added and the samples were run on the flow cytometer.

Flow cytometry to measure cluster size and apoptosis

Flow cytometry was similar to that described above in specific aim one. Flow cytometry was used to measure, percent apoptosis and cluster size, in order to look at variation within the t0 and t5 time points during the settling selection experiment.

CHAPTER FOUR

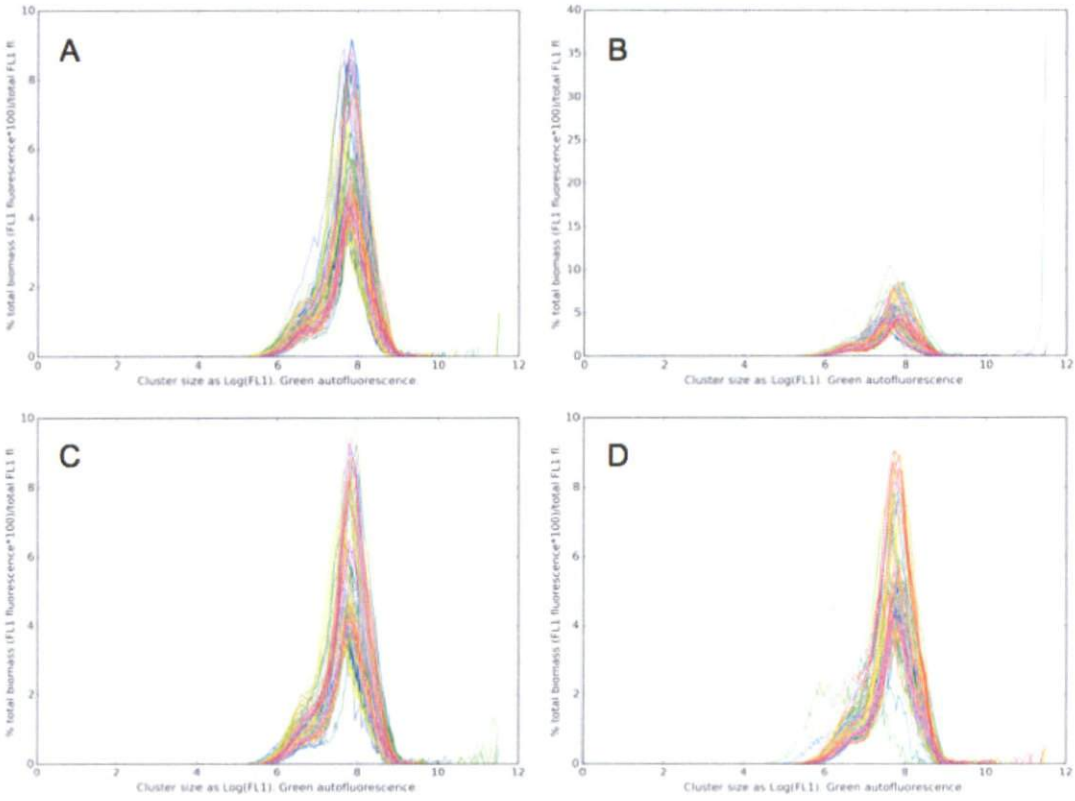
RESULTS

Specific aim one

Initial experiments were conducted using a flow cytometer to measure the cluster size. The intuitive way to measure cluster size is to take the average cluster size, but that is not an accurate measurement. The biomass mean is the correct way to measure the cluster size and not cluster level mean because the average of the distribution is smaller. Doing a biomass measurement allows for the cluster to be weighed and counted accordingly. Cells should not be counted evenly because some cells could weigh more than others, which causes undercounting in big groups and over counting in small groups.

After the 96 isogenic cultures for each population were grown, the flow cytometer collected the biomass data. From the data seen in figure 7, it is evident that sex does not create significant variation in the multicellular trait, size, with the exception of C1W8S. The 96 single-strain isolates at each point of evolution (C1W1-C1W6) have roughly the same distribution; there is a slight variation in the 96 isolates in the C1W8 population. Cluster size was measured as the log of FL1, or the green autofluorescence, and is represented on the x-axis. The percent biomass is represented on the y-axis; the equation, [(FL1 fluorescence x 100)/total FL1 fluorescence], is used to calculate the percent biomass. The cluster size was determined by the biomass mean; a biomass measurement

allowed us to weigh the cluster size and count them accordingly. The cells are not counted evenly when the cluster level mean is used because some clusters are larger than others, a problem we solve by weighting each cluster by its biomass. To avoid this, we calculate the cluster size that contains the mean amount of biomass (Figure 7) a weighted mean. The multipanel figure 7 represents the cluster size of each of the 96 strains isolated from the post-sex population of strains that have been undergoing selection for multicellularity for differing amounts of time (7-60 days); each color represents one of the 96 isogenic cultures at the specified time point. Figure 8 shows size data for eight replicates of each ancestral, pre-sex genotype. By comparing the figures, 7 and 8, of the post sex population and the pre-sex genotype, respectively, we determined sex did not increase variation in cluster size.



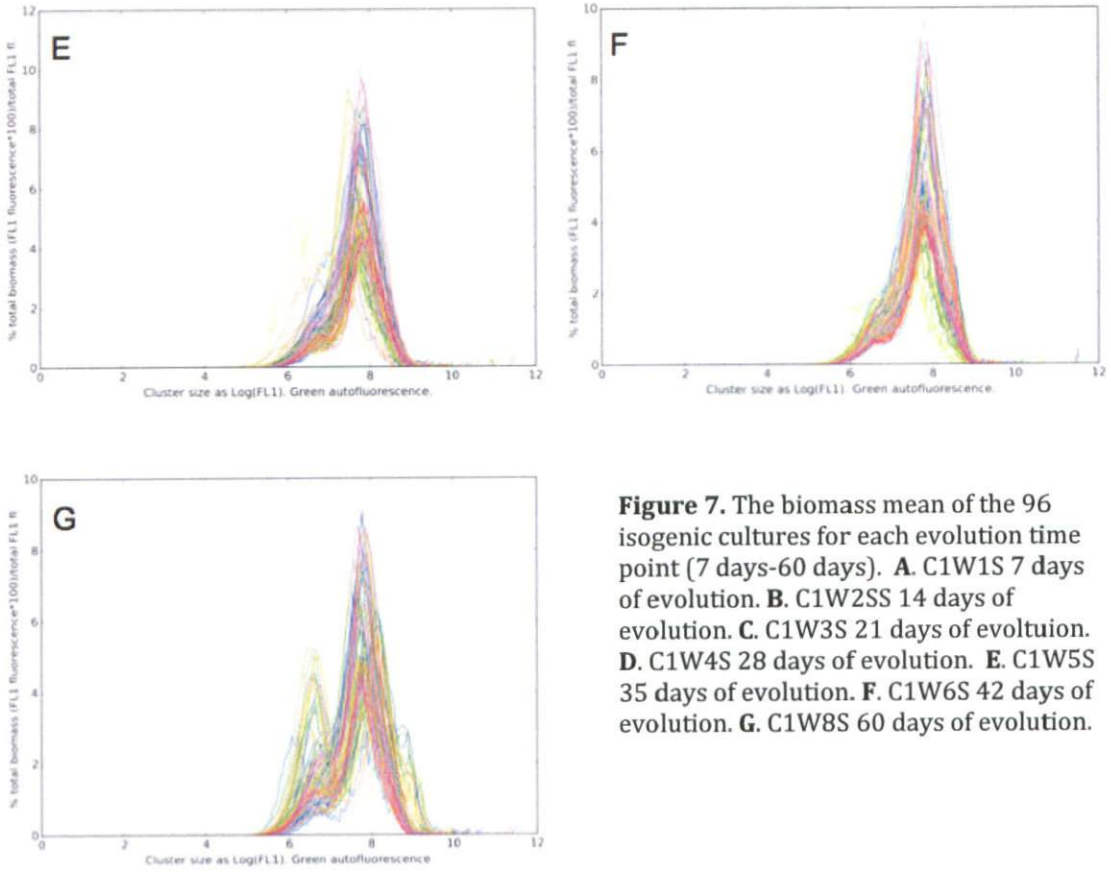


Figure 7. The biomass mean of the 96 isogenic cultures for each evolution time point (7 days-60 days). **A**. C1W1S 7 days of evolution. **B**. C1W2SS 14 days of evolution. **C**. C1W3S 21 days of evoltuion. **D**. C1W4S 28 days of evolution. **E**. C1W5S 35 days of evolution. **F**. C1W6S 42 days of evolution. **G**. C1W8S 60 days of evolution.

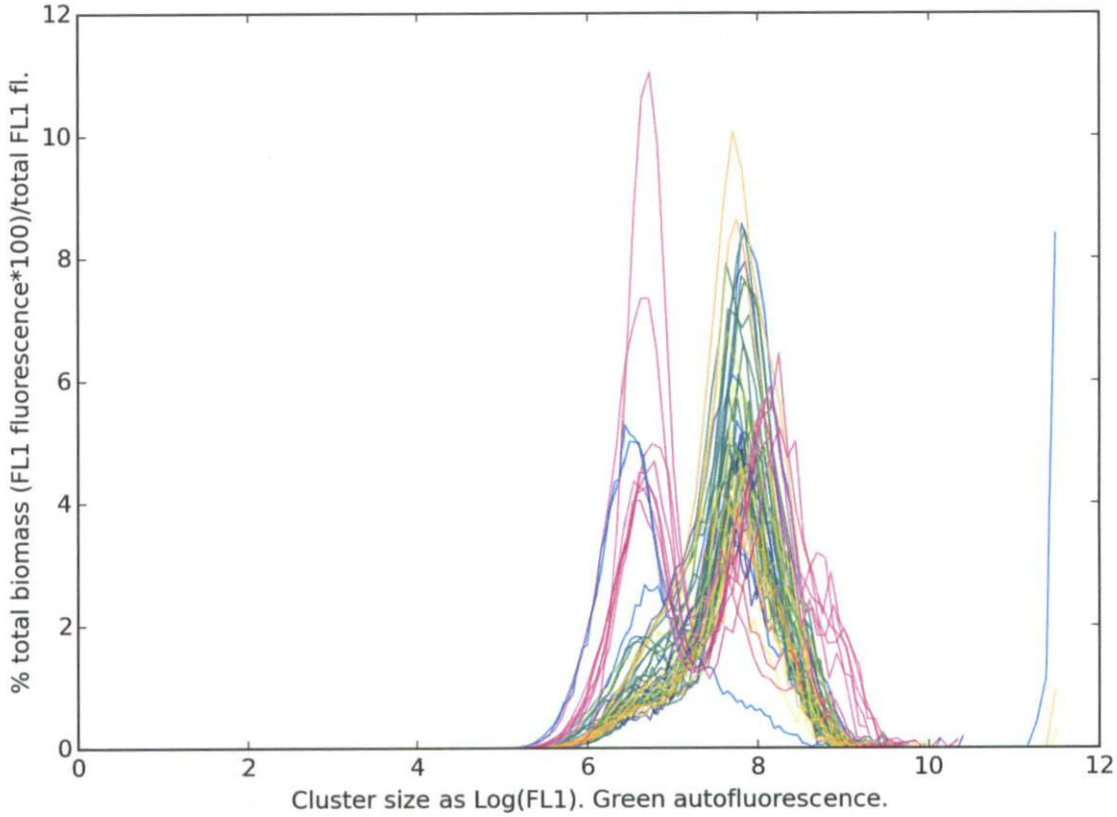


Figure 8. The biomass mean of the seven pre-sex ancestor strains (C1W1, C1W2, C1W3, C1W4, C1W5, C1W6, and C1W8) in each of the evolutionary time points (7-60days) for eight repetitions.

Apoptosis, or programmed cell death was another multicellular trait that was examined for variation in post-sex isolates. Propidium iodide (PI) was used to stain dead yeast cells. Ideally we would measure the proportion of apoptotic cells directly, but this is slow and laborious, and unsuitable for this high-throughput experiment. Here we use a proxy for apoptosis: the proportion of clusters that contain detectable levels of dead cells, a measure, which can be rapidly obtained using flow cytometry.

To measure the proportion of clusters with detectable amounts of PI stained dead cells, a MATLAB script was created to sector the population into those containing dead cells, and those without. Figure 9 is an example of the MATLAB script which creates a gate around the PI stained cells because the stained cells produced a higher amount of FL2 or orange fluorescence, than they should based on their green autofluorescence (FL1). Figure 10 is a multipanel comparison of the pre and post-sex isolates on their specified days of evolution. By comparing each of the images in figure 10, and comparing the days of evolution to each other, we concluded sex did not increase variation in apoptosis. Although in closer examination of figure 10 we determined W8 could be an outlier; the post-sex offspring were mostly lower in apoptosis than the parents, but some stayed high, so this population had an increase in variance, unlike the others.

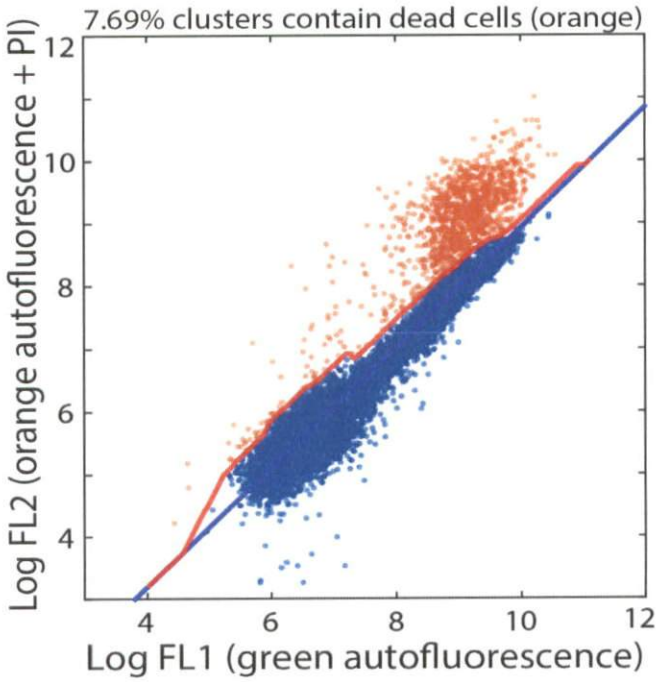


Figure 9.

A MATLAB script was created to automatically measure the effects of apoptosis. The red line separates clusters with detectable dead cells using propidium iodide (orange) from clusters without dead cells (blue).

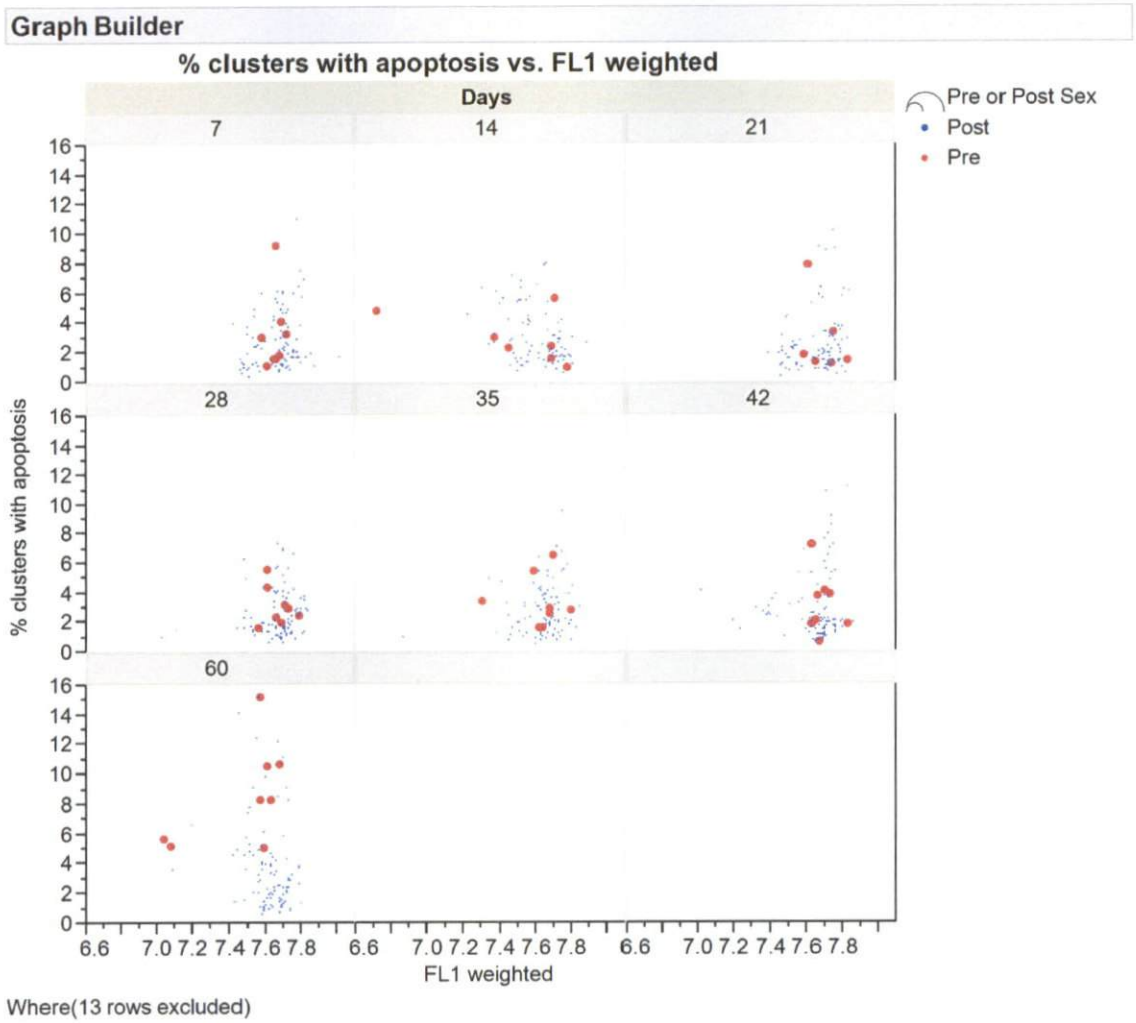
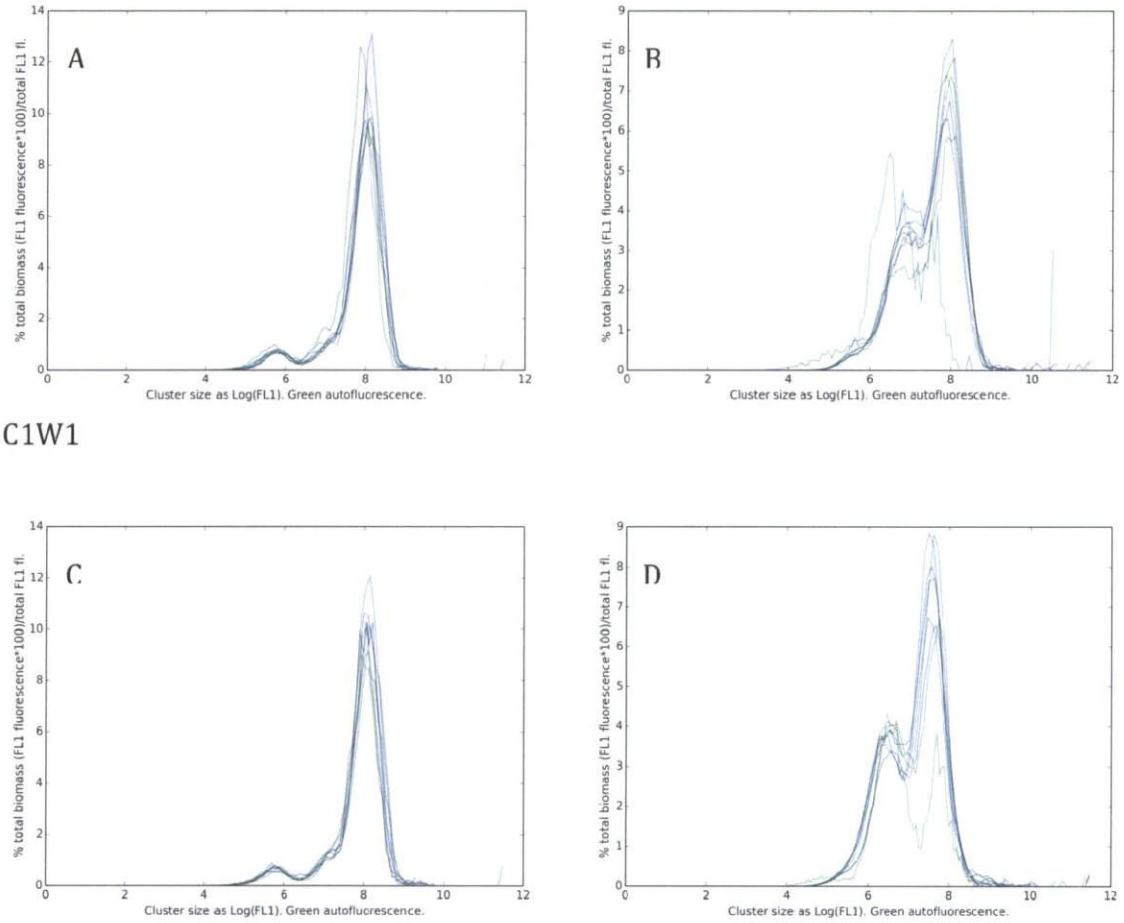


Figure 10. A multipanel depiction of pre and post sex isolates of the cluster size versus the percent of clusters within a cell that contain dead cells **(% apoptosis)**, which are separated by their days of evolution.

Specific aim two

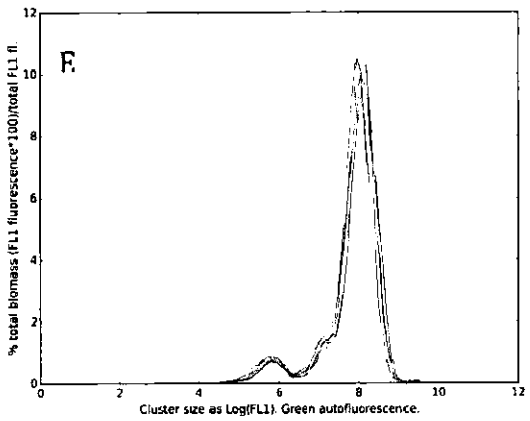
Settling selection was a contribution to the cluster size and apoptosis experiments. Settling selection added another perspective in determining what specific isogenic culture had the optimal fitness. Additionally, settling selection was performed on the pre-sex ancestors. The ancestors were measured at two specific points, T0 the first day of settling transfers and T5 the fifth day of settling transfers, but the populations were observed over the course of five days. These experiments were conducted to measure the cluster size and apoptosis of the timepoints T0 and T5, and to observe how the diverse populations changed over five transfers. From the data in figure 11, all populations decreased in size. There was a shift evident in the max peak cluster size; also, there was a significant increase in the biomass of small clusters as a whole and a decrease in the biomass of large clusters. The max cluster size seemed to decrease as the settling progressed. Figure 12 illustrated a shift from right to left in the average cluster size. This indicated that the size was reduced over the five-day experimental period (T0-T5), thus suggesting that there was little to no difference in apoptosis in the populations.

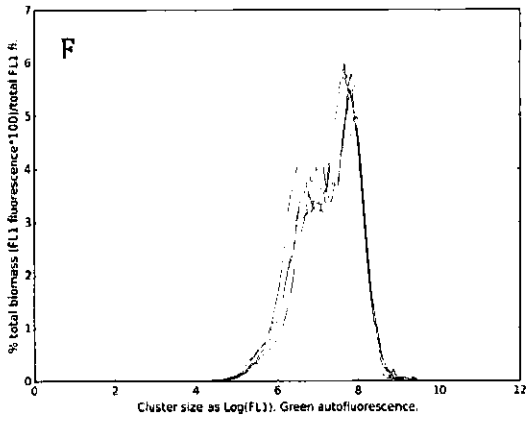


C1W1

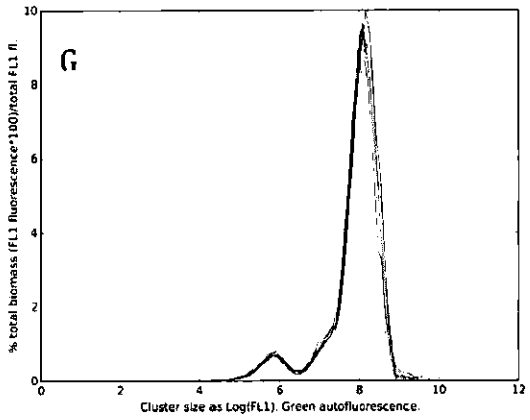
C1W2

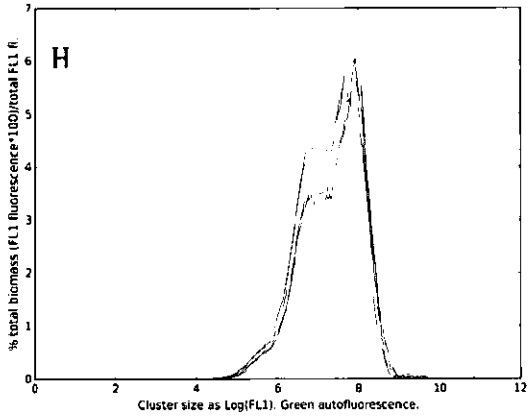
Figure 11. The settling selection experiment conducted over 5 days. A. T0 time point of C1W1S. B. T5 time point of C1W1S. C. T0 time point of C1W2S. D. T5 time point of C1W2S. E. T0 time point of C1W3S. F. T5 time point of C1W3S. G. T0 time point of C1W4S. H. T5 time point of C1W4S. I. T0 time point of C1W5S. J. T5 time point of C1W5S. K. T0 time point off C1W6S. L. T5 time point of C1W6S. M. T0 time point of C1W8S. N. T5 time point of C1W8S.



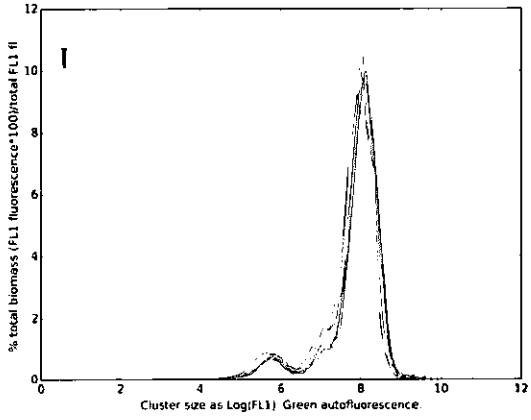


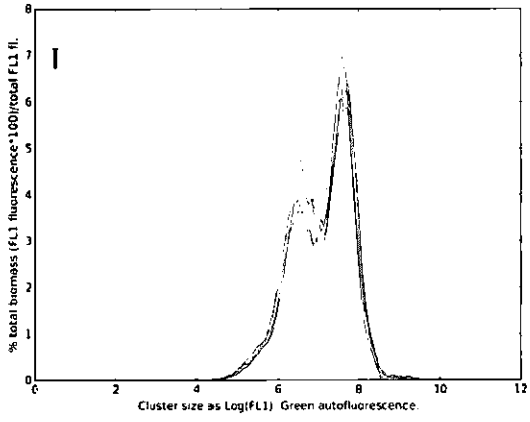
C1W3





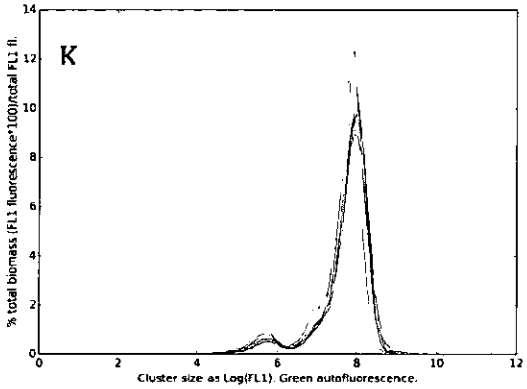
C1W4

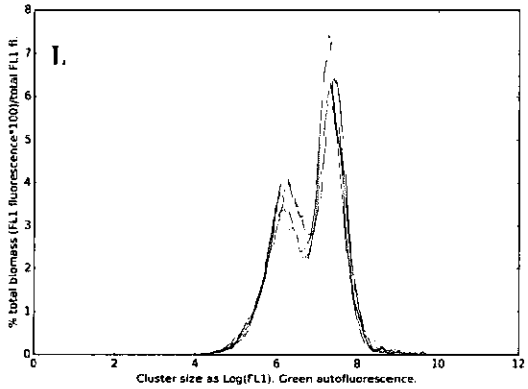




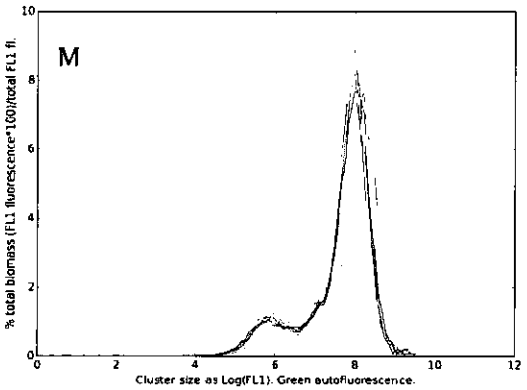
C1W5

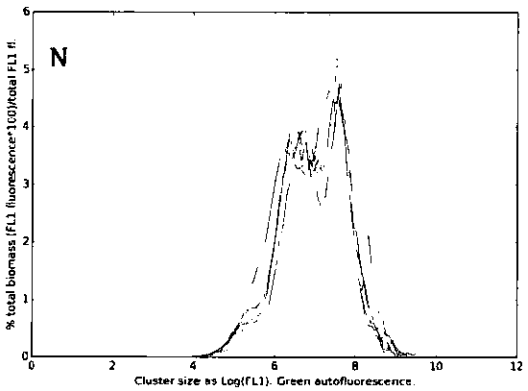
Figure 11. Continued





C1W6





C1W8

Figure 11. Continued

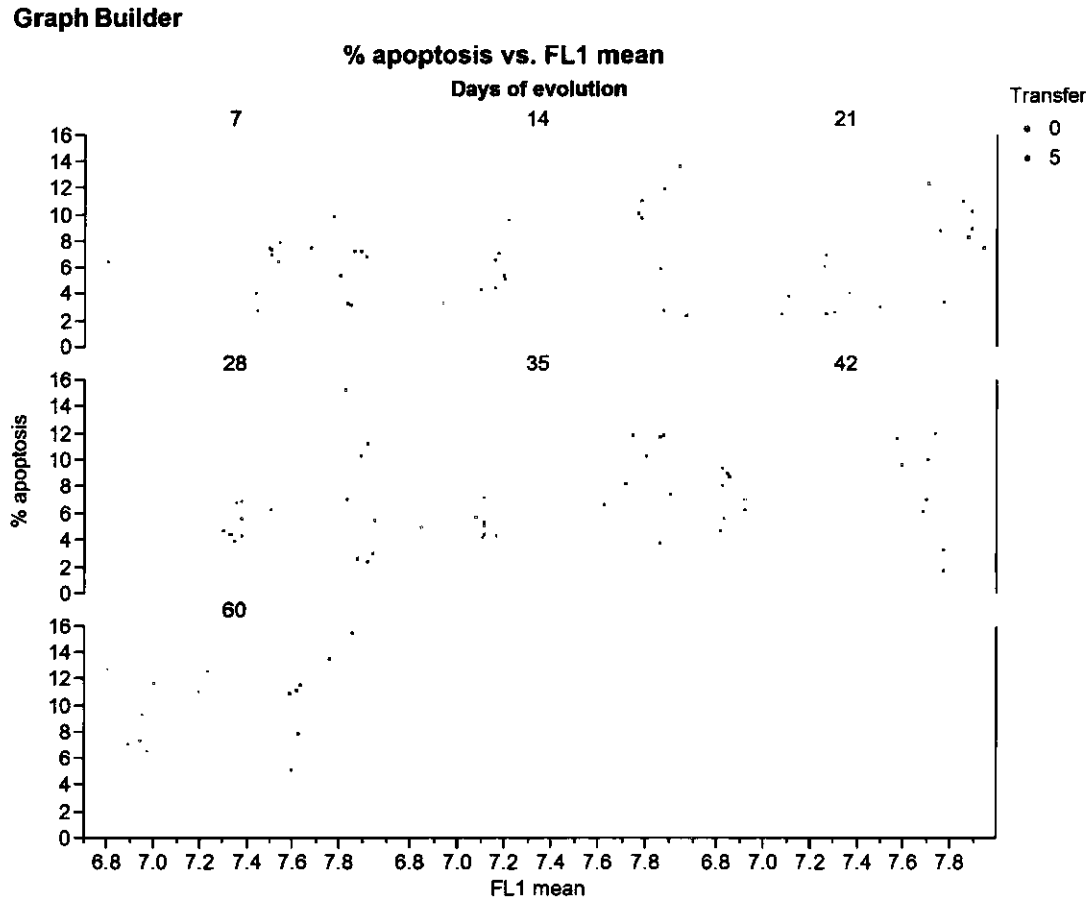


Figure 12. The settling selection experiment conducted over five days. Each multicellular trait, cluster size and apoptosis, was measured and compared on the first day of transfers (T0) and the last day of transfers (T5) over the 60 days evolutions. Cluster size is located on the on the x-axis, and percent apoptosis was on the y-axis.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

This study was conducted to determine if sex creates more variation in multicellular traits, allowing for rapid adaptation. The ability of S. *cerevisiae* to undergo sexual reproduction allows for the formation of new clusters, which exhibit different phenotypes. Sex in snowflake yeast appears to create smaller, lower apoptosis competitors that readily invade. It was not clear until after I completed my work that the experimental conditions I used may have actually been flawed, creating this effect. Snowflake yeast were originally evolved by centrifuging populations of yeast at 100 x g for 10 seconds, allowing large clusters to make it to the bottom of the tube and be passed on to fresh medium. Recently, others working in the Ratcliff lab discovered that 100 x g in their new centrifuge was not the same as 100 x g in their old centrifuge, and was effectively pelleting the entire population. As a result, there was no advantage to size, but yeast forming larger clusters grow less quickly than smaller ones, favoring smaller genotypes. If I were to redo this experiment, I would use settling selection conditions that have now been shown to reliably favor increased size, such as 5 minutes of settling at 1 x g.

The first set of experiments was used to observe the multicellular traits, size and percent apoptosis, in snowflake yeast. Once the data was collected it was evident that sex

did not create more variation of the size in the 96 isogenic cultures in their specific time point of evolution. In order to come to this conclusion, the more evolved isogenic cultures were compared to the less evolved pre-sex ancestor. The only semblance of variation was present on the 60th day of evolution; the post-sex ancestors exhibited lower levels of apoptosis when compared to the pre-sex ancestor.

The second set of experiments was used to compare the settling selection or how quickly larger clusters settled in comparison to smaller clusters from two different timepoints. The samples used were from the first day and fifth day of settling, the T0 and T5 timepoints, respectively. Settling selection was used to determine what specific combination of multicellular traits (size and apoptosis) had the highest fitness. Over the course of five days, the small clusters of snowflake yeast outcompeted the larger clusters and caused a significant shift in the cluster size. The large clusters were prominent on the first day of settling, but over time we saw an increase in the settling speed of the small clusters thereby increasing their fitness. This was due to the to the power of our centrifuge; in the previous Ratcliff 2012 experiments, the power of the centrifuge was significantly less than our current centrifuge. In our most recent experiments settling the population for 10 s on 100g caused pelleting of the entire population making size an insignificant factor; selection favored fast reproducing or small cluster. Apoptosis was also observed over the course of five days, and no significant shift was observed. This could indicate that our measure of apoptosis was not accurate for this experiment or that apoptosis was not under strong selection during this experiment.

Sex allows for the recombination of parental genetic material. Some of the combinations are essential to evolving an organism and increasing its fitness in its current environment. We can assume multicellularity evolved as a need for organisms to be larger. Because an advantage of multicellularity allows for the division of labor among cells in an organism, the ability of an organism to exhibit multicellularity is already increasing its fitness. While looking at seven populations at different timepoints during their evolution, size and apoptosis had little to no change from their pre-sex ancestor. Although cluster size and apoptosis didn't see an increase in variation due to sex during our early experiments, the variation increased after 60 days of evolution, this could indicate that the longer the organisms are evolving the more variation we may see. The combination of traits other than cluster size and apoptosis could be a reason for the increase in fitness in more highly evolved yeast. Due to the centrifuge, the settling selection experiment results were inconclusive. These results put us one step closer to determining which population has the optimal fitness. With more testing we will be able to determine the specific isogenic culture that has the highest relative fitness.

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